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Contribution to Confirmed & Synthesized on Mycorrhizae of *Tuber indicum* s.l. with Two Dominated & Subalpine Broadleaf Trees in Southwestern China

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Abstract

The ascomata and mycorrhizae of *Tuber indicum* s.l. were collected under the forest of broad-leaf species *Populus yunnanensis* and *Quercus pannosa* in the field respectively. The symbiotic relationships of both trees with *T. indicum* were examined and affirmed based on morphology and ITS-rDNA sequences. These two mycorrhizal combinations were successfully produced on artificially controlled substrates and cultural condition. This is the first report of a mycorrhizal association and synthesis between Chinese black truffles and poplars. A hyphal net covering the mantle's surface of the mycorrhizae was detected in both mycorrhizal combinations. The mycorrhizal colonization of *P. yunnanensis* and *Q. pannosa* suggests that *T. indicum* s.l. has a broader host range and that additional corresponding wood species would be used as candidates for the cultivation of *T. indicum*. The nuclear-ITS sequences of the mycorrhizae included in the phylogeny of the *T. indicum* complex revealed that the two clades within the complex do not markedly differ with respect to their preferences for host species or geographical origin. Our results help to explain the wide distribution of both clades of the *T. indicum* complex. It would be more important for truffle conservation and Chinese black truffle plantation development with these two dominated & alpestrine *Populus yunnanensis* and *Quercus pannosa* at subalpine limestone areas in China.

Keywords

Host Preference, *Populus yunnanensis*, *Quercus pannosa*, Mycorrhizae, Truffle, Conservation & Plantation

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1. Introduction

The Asian black truffle, *Tuber indicum* s.l., comprises a species complex that includes *T. indicum* Cooke & Massee, *T. himalayense* B. C. Zhang & Minter and *T. sinense* K. Tao & B. Liu and is commercially important both in China and Europe as a result of its introduction to Europe in the 1990s. This commercial importance has motivated a series of studies on the taxonomy and phylogeny of this complex. Because these fungi are ectomycorrhizal, investigations into the symbiotic relationships of the *T. indicum* complex with trees are of practical and scientific importance. Several tree species are thought to be natural hosts: *Quercus incana* Roxb. for *T. himalayense* in Himalayan India [1] and *Pinus armandii* Franch. and *Pi. yunnanensis* Franch. for *T. sinense* in southwestern China [2] [3]. In a previous study that tested the ability of *T. indicum* s.l. taxa to form mycorrhizae on artificially controlled substrates, eight Chinese trees in the genera *Quercus*, *Cyclobalanopsis*, *Carpinus*, *Pinus* and *Castanea* were successfully inoculated with *T. indicum* s.l. [4]-[7]. Successful mycorrhizal synthesis was also observed for *T. indicum* s.l. and the North America trees *P. taeda* L. and *Carya illinoinensis* (Wangenh.) K. Koch [8] and the European trees *Q. pubescens* Willd., *Q. cerris* L., *Q. ilex* L., *Corylus avellana* L. and *Pi. pinea* L. [10]-[13].

Almost two decades of inappropriate collection and excessive commercialization have seriously threatened the natural sources of *T. indicum* s.l. in southwestern China [14]. To protect *T. indicum* s.l. while also meeting the market demand, Chinese mycologists are attempting to establish truffle plantations by producing truffle-colonized seedlings. Plantations of *Cy. glauca* colonized with *T. formosanum*, a species closely related to *T. indicum* s.l. [15], were reported to yield truffles eight years after transplantation [16]. The successful cultivation of *T. formosanum* implies that *T. indicum* s.l. plantations are feasible. The truffle plantation of *Pinus armandii* Franch. & *Castanea mollissima* Blume infected with *T. indicum* respectively was set up in the spring of 2008, and begin to produce truffle fruit bodies fourth years after transplantation (http://www.cas.cn/ky/kyjz/201212/t20121220_3725060.shtml). To achieve the goal of establishing such plantations, the first step is to determine which trees can be used as its hosts, an analysis that necessitates a reliable field record of the symbiotic relationships between *T. indicum* s.l. and tree species. However, in China, most of the reports on the host trees of *T. indicum* s.l. have been based on natural habitats, not on mycorrhizae collection and observation. The morphological details of mycorrhizae are also necessary to determine the quality of *T. indicum* s.l.-inoculated seedlings. Up to now, only the mycorrhizae of *T. indicum* s.l. on *Castanea mollissima* BL. and *P. armandii* have been described in China [5]. The widespread distribution of *T. indicum* s.l. in southwestern China indicates that there are likely undiscovered mycorrhizal relationships. One objective of this work was to identify new natural hosts of *T. indicum* s.l. and to provide detailed descriptions of their mycorrhizae, and then to synthesis truffle mycorrhizae based on the results for the truffle conservation and plantation.

In recent years, phylogenetic studies have found that there are two phylogenetic clades within *T. indicum* s.l. [2] [9] [15] [17]-[23]. To explain this phylogenetic diversification, previous studies investigated the geographical and biological characteristics of the two clades. Using ITS-RFLP analysis, Paolocci *et al.* [20] suggested that geographical origin could contribute to the inter-clade variability. This hypothesis was supported by the study of Wang *et al.* [22]. However, after analyzing the origins, Zhang *et al.* [23] failed to find any correlation between phylogenetic relationships and either host or geographical origin. Chen *et al.* [15] obtained similar results, except that they found that *T. formosanum*, a small subclade within one of the two major clades in *T. indicum* s.l., should be treated as a separate species based on its exclusive association with *Cy. glauca*, its geographic distribution and its different morphology. Because all of the research was based on ascomata collected from markets or herbaria, the question about host preference or/and geographical contribution was unresolved. The objective of this work was to determine if there are differences in host preference or/and geographical range between different phylogenetic clades in the species complex of *T. indicum* by comparing the sequences of *T. indicum* s.l. mycorrhizae and ascomata collected *in situ* and look for new host trees.

2. Materials and Methods

2.1. Sample Collections

After collecting ascomata of *T. indicum* s.l. (the locations are listed in **Table 1**), the soil beneath the ascomata was removed until the mycorrhizae was visible. Approximately 30 mycorrhizal tips with soil were collected and stored in an ice box. All samples were transported to the laboratory within one week. The soil samples contain-

Table 1. Information of samples for ITS sequences used in molecular analysis.

Species name	Genbank No.	Source	Host	Locality
T. indicum s.l.	JQ638967	Natural mycorrhizae	*Po. yunnanensis*	Kunming, Yunnan
T. indicum s.l.	JQ638968	Natural mycorrhizae	*Po. yunnanensis*	Kunming, Yunnan
T. indicum s.l.	JQ638963	Natural mycorrhizae	*Pi. armandii*	Kunming, Yunnan
T. indicum s.l.	JQ638964	Natural mycorrhizae	*Pi. armandii*	Huize, Yunnan
T. indicum s.l.	JQ638965	Natural mycorrhizae	*Pi. armandii*	Huize, Yunnan
T. indicum s.l.	JQ638966	Natural mycorrhizae	*Pi. armandii*	Huize, Yunnan
T. indicum s.l.	JQ638971	Natural mycorrhizae	*Pi. armandii*	Dali, Yunnan
T. indicum s.l.	JQ638973	Natural mycorrhizae	*Pi. armandii*	Panzhihua, Sichuan
T. indicum s.l.	JQ638974	Natural mycorrhizae	*Pi. armandii*	Panzhihua, Sichuan
T. indicum s.l.	JQ638962	Natural mycorrhizae	*Pi. yunnanensis*	Kunming, Yunnan
T. indicum s.l.	JQ638969	Natural mycorrhizae	*Pi. yunnanensis*	Kunming, Yunnan
T. indicum s.l.	JQ638970	Natural mycorrhizae	*Pi. yunnanensis*	Kunming, Yunnan
T. indicum s.l.	JQ638972	Natural mycorrhizae	*Ca. mollissima*	Chuxiong, Yunnan
T. indicum s.l.	JQ638975	Natural mycorrhizae	*Q. pannosa*	Gongshan, Yunnan
T. indicum s.l.	JQ638976	Natural mycorrhizae	*Q. pannosa*	Gongshan, Yunnan
T. indicum s.l.	JQ638977	Synthesized mycorrhizae	*Pi. armandii*	
T. indicum s.l.	JQ638978	Synthesized mycorrhizae	*Pi. armandii*	
T. indicum s.l.	JQ638979	Synthesized mycorrhizae	*Pi. armandii*	
T. indicum s.l.	JQ638980	Synthesized mycorrhizae	*Pi. armandii*	
T. indicum s.l.	JQ638981	Synthesized mycorrhizae	*Pi. armandii*	
T. indicum s.l.	JQ638953	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638954	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638956	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638957	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638955	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638959	Synthesized mycorrhizae	*Ca. mollissima*	
T. indicum s.l.	JQ638984	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638985	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638986	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638987	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638988	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638989	Ascomata		Kunming, Yunnan
T. indicum s.l.	JQ638990	Ascomata		Huize, Yunnan
T. indicum s.l.	JQ638991	Ascomata		Huize, Yunnan
T. indicum s.l.	JQ638992	Ascomata		Huize, Yunnan
T. indicum s.l.	JQ638993	Ascomata		Huize, Yunnan
T. indicum s.l.	JQ638994	Ascomata		Chuxiong, Yunnan
T. indicum s.l.	JQ638995	Ascomata		Dali, Yunnan
T. indicum s.l.	JQ638996	Ascomata		Dali, Yunnan

Continued

T. indicum s.l.	JQ638997	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ638998	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ638999	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ639000	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ639001	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ639002	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ639003	Ascomata	Gongshan, Yunnan
T. indicum s.l.	JQ639004	Ascomata	Panzhihua, Sichuan
T. indicum s.l.	JQ639005	Ascomata	Panzhihua, Sichuan
T. indicum s.l.	JQ639007	Ascomata	Panzhihua, Sichuan
T. pseudexcavatum	JQ639006	Ascomata	Panzhihua, Sichuan
T. pseudexcavatum	JQ638958	Ascomata	Panzhihua, Sichuan
T. pseudexcavatum	JQ638982	Ascomata	Kunming, Yunnan
T. pseudexcavatum	JQ638983	Ascomata	Kunming, Yunnan
T. melanosporum	JQ638960	Ascomata	Italy
T. melanosporum	JQ638961	Ascomata	Italy
T. melanosporum	JQ639008	Ascomata	Italy
T. melanosporum	JQ639009	Ascomata	Italy

ing the mycorrhizae were placed in large dishes and soaked in water until the soil debris could be removed from mycorrhizae with needles. After being cleaned, the freshly isolated mycorrhizae were ready for morphological observation and DNA extraction. The host trees of the mycorrhizal samples used in this study is listed in **Table 1**.

2.2. Morphological Study

The macroscopic and microscopic characters of mycorrhizae were described and illustrated based on the descriptions of natural mycorrhizae by Agerer [24]. The mycorrhizae were photographed under a Nikon SMZ1500 stereoscope. Cross- and longitudinal sections of the mycorrhizae were prepared with a Leica CM1100 freezing microtome. The mantle layers and the anatomical structures of the hyphae were sectioned by hands. All anatomical sections were observed under a Nikon Eclipse E400 microscope and photographed with a Nikon E4500 camera.

2.3. DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from ascomata or 6 - 10 mycorrhizal tips using the CTAB method (Doyle, 1987 [25]) with the minor modification of adding 200 µL of 5 M potassium acetate after the treatment with 4 × CTAB. The ITS5 primers [26] and ITS4LNG [27] were used to amplify the ITS region from the ascomata or mycorrhizal samples to confirm the identities of the fungi in the mycorrhizae using blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The final amplification reactions (25 µL) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, 1.5 U of Taq Polymerase (Takara Taq, Takara Biotechnology, Dalian Co. Ltd., China), 0.2 µL of BSA (1%) and 50 ng of DNA template. The cycling parameters were an initial denaturation at 95˚C for 5 min; 35 cycles of denaturation at 94˚C for 1 min, annealing at 52˚C for 45 s and extension at 72˚C for 45 s; and a final extension at 72˚C for 10 min. The amplification products were electrophoresed on a 1.2% agarose gel. Sequencing was performed with an ABI Prism BigDye terminator cycle sequencing kit v3.1 and an ABI PRISM 3730 automatic sequencer.

2.4. Data Analysis

ITS sequences were edited with SeqMan (DNASTAR Package). Alignments were performed with Clustal X

Version 1.81 [28] and adjusted manually with BioEdit Version 5.0.9 [29]. Maximum parsimony analysis was conducted with Paup* 4.0b 10 [30] using a heuristic search and tree bisection reconnection (TBR) branch swapping with 1000 search replicates; a random sequence was added to each replicate. Gaps were treated as missing data. Character states were treated as equally weighted and unordered. Four ITS sequences of *T. pseudoexcavatum* G. Moreno *et al.* were used as the outgroup. To indicate the phylogenetic diversity within the *T. indicum* complex, 4 ITS sequences of the sibling species *T. melanosporum* Vittad. were included in the phylogenic analysis.

2.5. Mycorrhizal Synthesis

The fully mature ascocarps of *T. indicum* used in mycorrhizal synthesis were commercially acquired from a wild edible fungous market in Kunming, Yunnan, China, and the origins were unknown. Seeds of *Q. pannosa* provided by the Kunming Botanical Garden were used to produce seedlings following the method in [5]. The *Po. yunnanensis* seedlings were propagated by the traditional cutting & layering method. The methods for the production of *T. indicum* s.l. mycorrhizae on host trees and the examination of mycorrhizae followed the protocols in [5].

3. Results

3.1. Morphological Characters of Mycorrhizae

Tuber indicum s.l. × *Poplus yunnanensis* (**Figure 1**).

Mycorrhizal system unramified to monopodial-pinnate, with 1 - 2 orders of ramification, brown to light brown in color; short-distance exploration type (**Figure 1(a)**). Main axes 1 - 3.5 mm long, 0.7 - 1.5 mm in di-

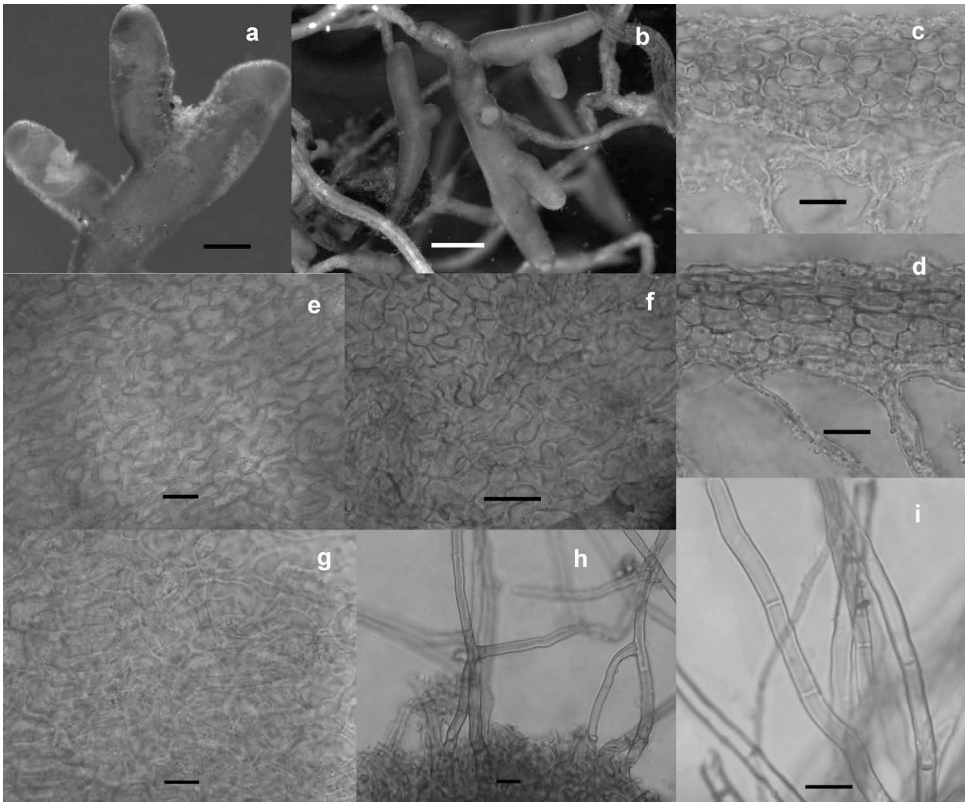

Figure 1. Mycorrhizae formed by *T. indicum* s.l. on *Po. yunnanensis.* (a) Natural mycorrhizae (bar = 200 μm); (b) Synthesized mycorrhizae (bar = 2000 μm); (c) Cross-section of mantle (bar = 10 μm); (d). Longitudinal section of mantle (bar = 10 μm); (e) Outer mantle layer (bar = 10 μm); (f) Inner mantle layer (bar = 10 μm); (g) Hyphal net on mantle (bar = 10 μm); (h)-(i) Emanating hyphae (bar = 10 μm).

ameter, straight or slightly bent. Unramified ends 0.5 - 3 mm long, 0.5 - 0.8 mm in diameter, straight, cylindrical or slightly tapering, apex round, tips usually lighter than the other parts (**Figure 1(b)**). Surface smooth or woolly with whitish emanating hyphae; emanating hyphae distinct, numerous, and distributed unequally.

Cross-section of mantle 15 - 25 µm thick, pseudoparenchymatous composed of 4 - 6 layers with elliptical to hyphal cells 3 - 13 × 2 - 8 µm, walls thin or slightly thick, light brown, slightly discernible with different layers (**Figure 1(c)**). Longitudinal section of mantle 18 - 25 µm thick, pseudoparenchymatous composed of 5 - 7 layers of elliptical to hyphal cells 4 - 20 × 2 - 8 µm, walls thin or slightly thick, light brown, subglobose to elliptic, slightly discernible with different layers (**Figure 1(d)**). The tip in transverse sections same as in other parts but with smaller cells. Hartig net reaches the 1 - 2 layers of cortex cells, composed of 1 - 2 layers of hyphal cells, colorless, subglobose, 1 - 3 µm in diameter.

Mantle in plan views is covered by a hyphal network, especially in regions of growth (**Figure 1(g)**). The surface net 1 - 2 layers, formed by anastomosed and irregularly connected hyphae, 3 - 5 µm in diameter, 5 - 12 µm between septa, cell walls thin to slightly thick, smooth, colorless to yellowish brown. Outer mantle layers pseudoparenchymatous epidermoid with irregularly polygonal and sinuous cells interlocked in a puzzle-like pattern, cells 8 - 25 × 4 - 10 µm, 6 - 8 cells in a square of 20 × 20 µm, thick walled, with yellowish brown walls, 1 - 2 µm thick, not gelatinous (**Figure 1(e)**). Inner mantle layers pseudoparenchymatous or a transitional type between plectenchymatous and pseudoparenchymatous, cells 7 - 18 × 3 - 10 µm, 6 - 9 cells in a square of 20 × 20 µm, thin walled or slightly thick walled (**Figure 1(f)**). Mantle structures of the very tips same as in other parts of the mycorrhizae but with smaller cells.

Emanating hyphae growing from hyphal net, loosely woolly, yellowish brown to blackish brown, cylindrical, tips rounded or tapering, thin walled, smooth, straight or bent, 25 - 280 µm long, 2 - 4 µm in diameter at the top, 3 - 5 µm in diameter at the base, septate, 20 - 80 µm long between two septa, ramifications frequent and almost perpendicular branching near base, clamps lacking (**Figure 1(h)** and **Figure 1(i)**).

Tuber indicum s.l. × *Quercus pannosa* (**Figure 2**).

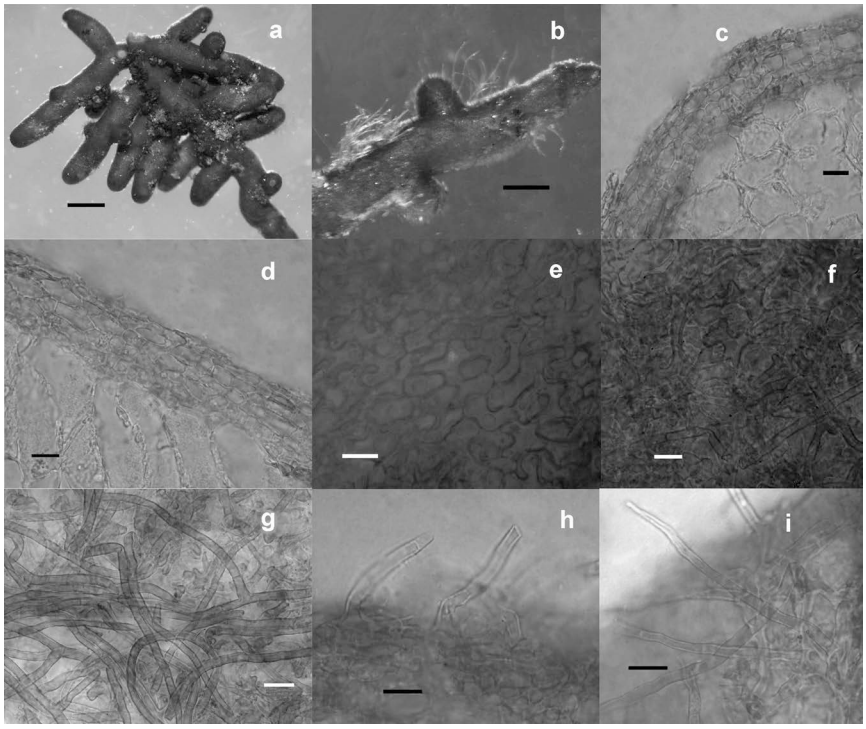

Figure 2. Mycorrhizae formed by *T. indicum* s.l. on *Q. pannosa*. (a) Type of ramification (bar = 500 µm); (b) Mycorrhizal tips with emanating hyphae (bar = 150 µm); (c) Cross-section of the mantle (bar = 10 µm); (d) Longitudinal section of the mantle (bar = 10 µm); (e) Mantle in plan views (bar = 10 µm); (f) Hyphal net on the mantle (bar = 10 µm); (g) Emanating hyphae in plan views (bar = 10 µm); (h) & (i) Emanating hyphae in transverse views (bar = 10 µm).

Mycorrhizal system unramified, monopodial-pinnate to monopodial-pyramidal, with 1 - 3 orders of ramification, light brown when young, brown to darkish brown; short-distance exploration type (**Figure 2(a)**). Main axes 0.8 - 3.5 mm long, 0.3 - 0.5 mm in diameter, straight or slightly bent. Unramified ends 0.3 - 2 mm long, 0.2 - 0.5 mm in diameter, straight or rarely slightly bent, cylindrical or slightly tapering, apex round (**Figure 2(b)**). Surface smooth or woolly with whitish emanating hyphae; emanating hyphae distinct, numerous, distributed unequally.

Cross-section of mantle 12 - 25 µm thick, pseudoparenchymatous composed of 4 - 7 layers of elliptical to hyphal cells, cells 3 - 15 × 2 - 6 µm, walls thin or slightly thick, indiscernible with different layers (**Figure 2(c)**). Longitudinal section of mantle 18 - 25 µm thick, pseudoparenchymatous composed of 5 - 7 layers of subglobose hyphal cells; cells 3 - 25 × 2 - 6 µm, walls thin or slightly thick, indiscernible with different layers (**Figure 2(d)**). The tip in transverse sections same as in other parts but with smaller cells. Hartig net reaches the 1 - 2 layers of cortex cells, composed of 1 - 2 layers of hyphal cells, colorless, subglobose, 1 - 3 µm in diameter.

Mantle in plan views is covered by a hyphal network, especially in regions of growth (**Figure 2(f)**). Surface net 1 - 3 layers, formed by anastomosed and irregularly connected hyphae, 3 - 8 µm in diameter, 6 - 12 µm between septa, cell walls thin to slightly thick, smooth, colorless to yellowish brown. Mantle indiscernible with different layers, composed of pseudoparenchymatous epidermoid with irregularly polygonal cells interlocked in a puzzle-like pattern, cells 8 - 25 × 5 - 15 µm, 4 - 8 cells in a square of 20 × 20 µm, thick walled, with pale brown walls, 0.5 - 1.5 µm thick, not gelatinous (**Figure 2(e)**). Mantle structures of the very tips same as in other parts of mycorrhizae but with smaller cells.

Emanating hyphae growing from both the outer mantle layer cells and hyphal net, loosely woolly, whitish to yellowish brown, cylindrical, tips rounded, thin walled, smooth, straight or bent, 60 - 350 µm long, 2 - 3 µm in diameter at the top, 3 - 4 µm in diameter at the base, septate, 20 - 60 µm long between two septa, ramifications frequent and almost 60 - 90 degree branching, clamps lacking (**Figures 2(g)-(i)**).

3.2. Phylogenetic Analysis

Forty-nine ITS sequences of *T. indicum* s.l. were used in the phylogenetic analysis (**Table 1**), including 23 from ascomata, 15 from natural mycorrhizae and 11 from synthesized mycorrhizae. Of the 566 total characters analyzed, 371 characters were constant, 18 were parsimony uninformative, and 177 were parsimony informative. The most parsimonious tree (**Figure 3**) included 261 steps (CI = 0.8697, RI = 0.9693).

The ITS phylogenetic tree (**Figure 3**) revealed that *T. indicum* s.l. is well separated into Group A and Group B. Two subclades, Group A and *T. melanosporum*, cluster in one clade that is a sister Group to with Group B with 100% bootstrap support. Thirty-one sequences of *T. indicum* s.l. clustered in Group A, and 18 clustered in Group B (the geographic distribution of the two groups is shown in **Figure 4**). Eighteen ITS sequences in Group A and 8 ITS sequences in Group B were from mycorrhizae (sequence origins are shown in **Table 1**).

4. Discussion

Using blast and ITS sequences from natural mycorrhizae in the NCBI database, we confirmed the symbiotic relationships between *T. indicum* s.l. and *Q. pannosa*, *Po. yunnanensis*, *Ca. mollissima*, *Pi. armandii* and *Pi. yunnanensis*. All *Q. pannosa* and *Po. yunnanensis* seedlings formed mycorrhizal systems with *T. indicum* on artificially controlled substrates. Before this study, eight indigenous trees of the genera *Quercus*, *Cyclobalanopsis*, *Castanea*, *Carpinus* and *Pinus* were successfully inoculated with *T. indicum* s.l. [4]-[7]. *Tuber indicum* s.l. has also been reported to form mycorrhizae with two North American trees, *Pi. taeda* and *Carya illinoinensis* [8], and five European trees, *Q. pubescens*, *Q. cerris*, *Q. ilex*, *Co. avellana* and *Pi. pinea* [10]-[13] under artificially controlled conditions. Currently, sixteen trees are known to be symbiotic with *T. indicum* s.l. The higher diversity of the hosts indicates that *T. indicum* s.l. has wider host compatibility and higher ecological adaptability, suggesting that this species is more suitable than *T. menosporum* for artificial cultivation.

For the first time, we found Chinese black truffle mycorrhizae on poplars. Prior this study, the white truffles *T. magnatum* Pico and *T. rapaeodorum* Tul. & C. Tul. were found in association with the European poplar tree *Po. alba* L. [31]-[33]. The confirmation of the mycorrhizal relationship between *T. indicum* s.l. and *Po. yunnanensis* is of particular practical importance because Yunnan aspen seedlings can be easily produced by asexual reproduction and grow more rapidly. The Yunnan aspen is widely distributed in the middle-lowlands (altitude 1600 - 3500 m) of southwestern China and is an important species for afforestation because it grows quickly [34]. The

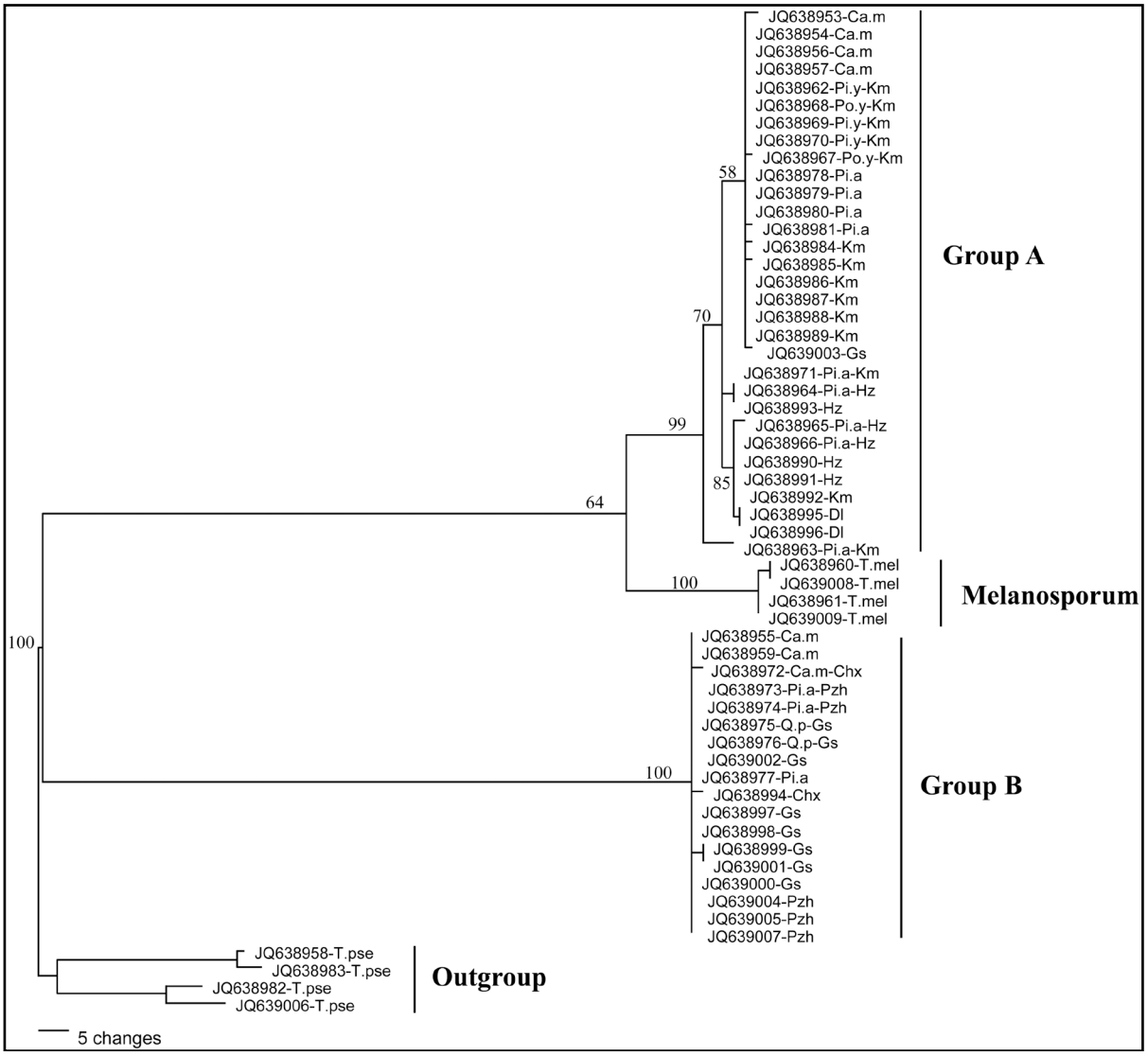

Figure 3. One of the 32 most parsimonious trees obtained from the analysis of the ITS sequences. (Km = Kunming, Gs = Gongshan, Hz = Huize, Dl = Dali, Chx = Chuxiong, Pzh = Panzhihua).

successful production of mycorrhizae using *Po. yunnanensis* seedlings and *T. indicum* s.l. confirmed the feasibility of using this tree on truffle conservation and plantations.

Tuber indicum s.l. mycorrhizae are characterized by a mantle composed of epidermoid cells interlocked in a puzzle-like pattern and woolly emanating hyphae that branch almost perpendicularly [5] [10]-[13] [35]. The mycorrhizae of *T. melanosporum*, a member of the same phylogenetic lineage, share all these characters with *T. indicum* s.l. except the hyphal net on the surface of the mantle, as shown in previous studies [36] [37]. However, a surface hyphal net on the mantle was also detected in *T. indicum* s.l. mycorrhizae in our study. These newly discovered characters broaden the diversity of *T. indicum* s.l. mycorrhizae, making it impossible to distinguish the mycorrhizae of these two species.

Geographical origins have been used to explain the inter-clade variability in *T. indicum* s.l. [20] [22]. The samples from Kunming, Huize, Dali and Gongshan, including *T. indicum* s.l. mycorrhizae and ascomata collected *in situ*, belonged to Group A, whereas samples from Gongshan, Panzhihua and Chuxiong belonged to Group B (shown in **Figure 4**). Groups A and B in our study are comparable to clades I and II, respectively, described by Wang *et al.* [22]. However, the origins of the two separate clades determined by Wang *et al.* [22] are different from the origins determined in our study. Samples from Gongshan all belonged to clade II in Wang *et*

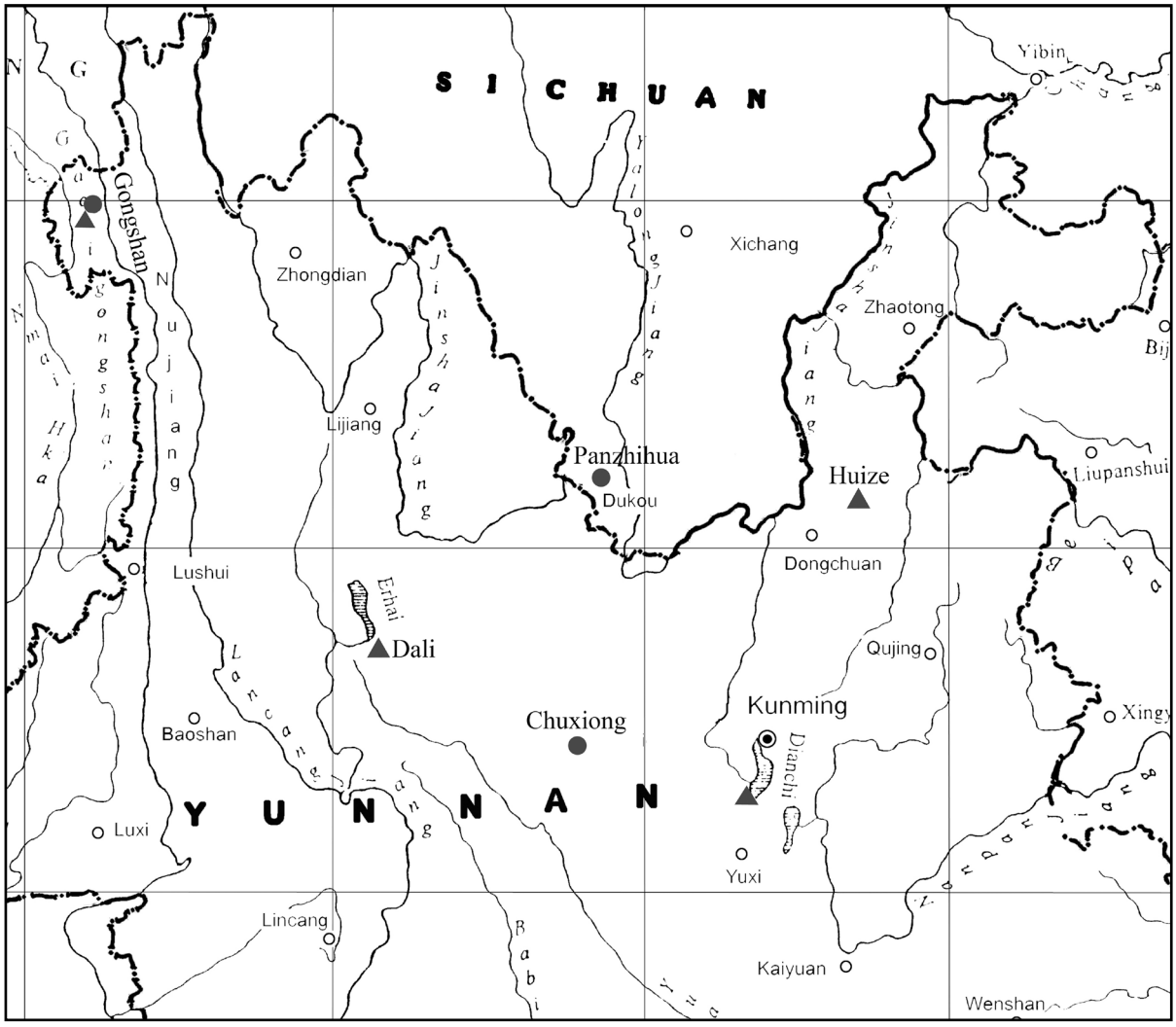

Figure 4. Geographic distribution of the two groups within *T. indicum* s.l. (triangles indicate the origins of Group A; circles indicate the origins of Group B).

al.'s study [22] but belonged to both Group A and Group B in our study. In addition, samples from Huize belonged to Group A and those from Chuxiong belonged to Group B in our study, whereas samples from Huize belonged to clade II and those from Chuxiong belonged Group I in Wang *et al.*'s study [22]. Because the samples were collected *in situ* in this study, our results indicate that two groups are not geographically distinct, supporting the results of Zhang *et al.* [23] and Chen *et al.* [15]. In our study, *Pi. armandii* was the natural host of five samples in Group A and of two samples in Group B, indicating that the two groups do not have distinct natural host preferences. Moreover, no host preference in the inoculation experiments was detected for Group A or Group B, as both groups included ITS sequences from mycorrhizae on *Pi. armandii* and *Ca. mollissima.* Although only ITS rDNA sequence was used in our study, the two clades in our phylogenic tree are consistent with the topologies inferred from ITS, LSU and *β*-tubulin sequences in Chen *et al.*'s study [14]. Our results support Chen *et al.*'s conclusion [15] that there are two cryptic species in the *T. indicum* complex. Furthermore, interclade differences in mycorrhizal morphology were not detected in our study, and the lack of distinct mycorrhizal morphologies could contribute to the presence of two cryptic species in the *T. indicum* complex.

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References

[1] Zhang, B.C. and Minter, D.W. (1988) *Tuber himalayense* sp. nov. with Notes on Himalayan Truffles. *Transactions of the British Mycological Society*, **91**, 593-597. http://dx.doi.org/10.1016/S0007-1536(88)80032-9

[2] Chen, J. (2007) Taxonomy and Phylogeny of the Genus *Tuber* in China. Ph.D. Dissertation, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 81-103.

[3] Zhang, D.C. and Wang, Y. (1990) Study on Chinese Truffle and Its Ecology. *Edible Fungi of China*, **9**, 25-27.

[4] Chen, B.T. (2003) Inoculation and Infection of *Tuber* spp. and Establishment of Truffle Plantation. *Guizhou Forestry Science and Technology*, **31**, 10-14.

[5] Geng, L.Y., Wang, X.H., Yu, F.Q., Deng, X.J., Tian, X.F., Shi, X.F., Xie, X.D., Liu, P.G. and Shen, Y.Y. (2009) Mycorrhizal Synthesis of *Tuber indicum* with Two Indigenous Hosts, *Castanea mollissima* and *Pinus armandii*. *Mycorrhiza*, **19**, 461-467. http://dx.doi.org/10.1007/s00572-009-0247-0

[6] Hu, B.F., Zhu, Z.R., Yuan, X.M., Yin, X.Y., Zhang, X.M., Wang, L., Fang, S., Yang, S. and Liao, W.B. (2004) The mycorrhizal Synthesis of *Tuber indicum* and Its Affect for Growth and against Disease on the Seedlings. *Guizhou Forestry Science and Technology*, **32**, 19-24.

[7] Hu, B.F., Yun, X.Y., Zhu, Z.R., Yuan, X.M., Yang, A.M., Jin, T.X., Hu, G., Liao, W.B. and Yang, P. (2006) Study on the Inoculation Technology of *Tuber sp.* for the Mycorrhizal Seedlings. *Guizhou Forestry Science and Technology*, **34**, 15-18.

[8] Bonito, G.M., Trappe, J.M., Donovan, S. and Vilgalys, R. (2011) The Asian Black Truffle *Tuber indicum* Can Form Ectomycorrhizas with North American Host Plants and Complete Its Life Cycle in Non-Native Soils. *Fungal Ecology*, **4**, 83-93. http://dx.doi.org/10.1016/j.funeco.2010.08.003

[9] Bonito, G.M., Gryganskyi, A.P., Trappe, J.M. and Vilgalys, R. (2010) A Global Meta-Analysis of *Tuber* ITS rDNA Sequences: Species Diversity, Host Associations and Long-Distance Dispersal. *Molecular Ecology*, **19**, 4994-5008. http://dx.doi.org/10.1111/j.1365-294X.2010.04855.x

[10] Comandini, O. and Pacioni, G. (1997) Mycorrhizae of Asian Black Truffles, *Tuber himalayense* and *T. indicum*. *Mycotaxon*, **63**, 77-86.

[11] Douet, J.P., Castroviejo, M., Mabru, D., Chevalier, G., Dupré, C., Bergougnoux, F., Ricard, J.M. and Médina, B. (2004) Rapid Molecular Typing of *Tuber melanosporum*, *T. brumale* and *T. indicum* from Tree Seedlings and Canned Truffles. *Analytical and Bioanalytical Chemistry*, **379**, 668-673. http://dx.doi.org/10.1007/s00216-004-2643-9

[12] García-Montero, L.G., Di Massimo, G., Manjón, J.L. and García-Abril, A. (2008) New Data on Ectomycorrhizae and Soils of the Chinese Truffles *Tuber pseudoexcavatum* and *Tuber indicum*, and Their Impact on Truffle Cultivation. *Mycorrhiza*, **19**, 7-14. http://dx.doi.org/10.1007/s00572-008-0198-x

[13] Zambonelli, A., Tibiletti, E. and Pisi, A. (1997) Caratterizzazione Anatomo-Morfologica Delle Micorrize di *Tuber indicum* Cooke & Massee su *Pinus pinea* L. e *Quercus cerris* L. *Micologia Italiana*, **26**, 29-36.

[14] Wang, Y. and Liu, P.G. (2009) Achievements and Challenges of Research on Truffles in China. *Acta Botanica Yunnanica*, **16**, 1-9.

[15] Chen, J., Guo, S.X. and Liu, P.G. (2011) Species Recognition and Cryptic Species in the *Tuber indicum* Complex. *PLoS ONE*, **6**, 1-10. http://dx.doi.org/10.1371/journal.pone.0014625

[16] Hu, H.T., Wang, Y. and Hu, B.Y. (2005) Cultivation of *Tuber formosanum* on Limed Soil in Taiwan. *New Zealand Journal of Crop and Horticultural Science*, **33**, 363-366. http://dx.doi.org/10.1080/01140671.2005.9514371

[17] García-Montero, L.G., Díaz, P., Di Massimo, G. and García-Abril, A. (2010) A Review of Research on Chinese *Tuber* Species. *Mycological Progress*, **9**, 315-335. http://dx.doi.org/10.1007/s11557-009-0647-8

[18] Huang, J.Y., Hu, H.T. and Shen, W.C. (2009) Phylogenetic Study of Two Truffles, *Tuber formosanum* and *Tuber furfuraceum*, Identified from Taiwan. *FEMS Microbiology Letters*, **294**, 157-171. http://dx.doi.org/10.1111/j.1574-6968.2009.01571.x

[19] Mabru, D., Dupré, C., Douet, J.P., Leroy, P., Ravel, C., Ricard, J.M., Medina, B., Castroviejo, M. and Chevalier, G. (2001) Rapid Molecular Typing Method for the Reliable Detection of Asiatic Black Truffle (*Tuber indicum*) in Commercialized Products: Fruiting Bodies and Mycorrhizal Seedlings. *Mycorrhiza*, **11**, 89-94. http://dx.doi.org/10.1007/s005720100103

[20] Paolocci, F., Rubini, A., Granetti, B. and Arcioni, S. (1997) Typing *Tuber melanosporum* and Chinese Black Truffle Species by Molecular Markers. *FEMS Microbiology Letters*, **153**, 255-260. http://dx.doi.org/10.1111/j.1574-6968.1997.tb12582.x

[21] Roux, C., Delmas-Sejalon, N., Martins, M., Parguey-Leduc, A., Dargent, R. and Bécard, G. (1999) Phylogenetic Relationships between European and Chinese Truffles Based on Parsimony and Distance Analysis of ITS Sequences. *FEMS Microbiology Letters*, **180**, 147-155. http://dx.doi.org/10.1111/j.1574-6968.1999.tb08789.x

[22] Wang, Y.J., Tan, Z.M., Zhang, D.C., Murat, C., Jeandroz, S. and Le Tacon, F. (2006) Phylogenetic and Populational Study of the *Tuber indicum* Complex. *Mycological Research*, **110**, 1034-1045. http://dx.doi.org/10.1016/j.mycres.2006.06.013

[23] Zhang, L.F., Yang, Z.L. and Song, D.S. (2005) A Phylogenetic Study of Commercial Chinese Truffles and Their Allies: Taxonomic Implications. *FEMS Microbiology Letters*, **245**, 85-92. http://dx.doi.org/10.1016/j.femsle.2005.02.028

[24] Agerer, R. (1987-2002) Color Atlas of Ectomycorrhizae. Einhorn-Verlag, Schwäbisch Gmünd.

[25] Doyle, J.J. and Doyle, J.L. (1987) A Rapid DNA Isolation Procedure from Small Quantities of Fresh Leaf Tissues. *Phytochemical Bulletin*, **19**, 11-15.

[26] White, T.J., Bruns, T.D., Lee, S.B. and Taylor, J.W. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds., *PCR Protocols*: *A Guide to Methods and Applications*, Academic Press, New York, 315-322. http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1

[27] Paolocci, F., Rubini, A., Granetti, B. and Arcioni, S. (1999) Rapid Molecular Approach for a Reliable Identification of *Tuber* spp. Ectomycorrhizae. *FEMS Microbiology Ecology*, **28**, 23-30. http://dx.doi.org/10.1111/j.1574-6941.1999.tb00557.x

[28] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X Windows Interface: Flexible Strategies for Multiple Sequence Alignment Aided by Quality Analysis Tools. *Nucleic Acids Research*, **25**, 4876-4882. http://dx.doi.org/10.1093/nar/25.24.4876

[29] Hall, T.A. (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

[30] Swofford, D.L. (2002) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), 4.0b4a. Sinauer Associates, Sunderland, Massachusetts.

[31] Angelini, P. and Granetti, B. (1995) La micorrizazione di alcuni cloni micropropagati di *Populus alba* L. con *Tuber magnatum* Pico. *Giornale Botanico Italiano*, **129**, 1161-1177. http://dx.doi.org/10.1080/11263509509436469

[32] Granetti, B. (1995) Caratteristiche morfologiche, biometriche e strutturali delle micorrize di *Tuber* di interesse economico. *Micologia Italiana*, **2**, 101-117.

[33] Jakucs, E. (2002) Ectomycorrhizae of *Populus alba* L. in South Hungary. *Phyton-Annales Rei Botanicae*, **42**, 199-210.

[34] He, C.Z., Che, P.Y., Zhou, X.T., Duan, A.A., Wang, D.X. and Xin, P.R. (2010) A Survey of Research Progress on Gene Resources of *Populus yunnanens*. *Journal of Southwest Forestry University*, **30**, 83-88.

[35] Hu, H.T. (1992) *Tuber formosanum* sp. nov. and Its Mycorrhizal Associations. *Journal of the Experimental Forest*, National Taiwan Univesity, **6**, 79-86.

[36] Pérez, F., Palfner, G., Brunel, N. and Santelices, R. (2007) Synthesis and Establishment of *Tuber melanosporum* Vitt. Ectomycorrhizae on Two *Nothofagus* Species in Chile. *Mycorrhiza*, **17**, 627-632. http://dx.doi.org/10.1007/s00572-007-0140-7

[37] Rauscher, T. and Chevalier, G. (1995) *Tuber melanosporum*. In: Agerer, R., Ed., *Colour Atlas of Ectomycorrhizae*, *Plate* 87, Einhorn-Verlag, Schwäbisch Gmünd.

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